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Expression cloning and antigenic analysis of the nucleocapsid protein of equine arteritis virus

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Abstract

A series of recombinant fusion proteins derived from equine arteritis virus (EAV) open reading frame (ORF) 7 have been used to define the immunoreactive region of the viral nucleocapsid (N) protein. Reactivities of recombinant N fusion proteins with post-infection equine sera in immunoblots and ELISAs indicate that the major nucleocapsid protein epitope is located within amino acid residues 1-69. In ELISAs two recombinant nucleocapsid fusion proteins containing residues 1-69 (rN1-69) and 1-28 (rN1-28) discriminated between pre- and post-infection, and pre- and post-vaccination serum samples. Additionally rN1-69 and rN1-28 detected seroconversions following vaccination with a killed virus preparation, even in the absence of a detectable virus neutralising response. Although a good correlation existed between virus neutralising antibody and rN1-69 ELISA positive values in post-infection sera, all the rN proteins failed to induce any virus neutralising response in immunised rabbits.

Keywords: Arterivirus; Equine arteritis virus (EAV); Nucleocapsid (N) protein; Antigenic analysis; Diagnostic antigen

1. Introduction

Equine viral arteritis (EVA) is a debilitating disease in horses (Chirnside, 1992) which at its most severe can cause abortion (Doll et al., 1957) and foal death (Golnik et al., 1981; Vaala et al., 1992). Diagnosis is complicated because the

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symptoms of infection are extremely varied and because disease outbreaks may remain clinically unapparent, only coming to light following serosurveys of equine populations. Virus transmission is by both the venereal and respiratory routes, with between 30 and 60% of seropositive stallions permanently shedding equine arteritis virus (EAV) in their semen following infection (Timoney et al., 1986). Shedding stallions used in breeding have initiated the spread of EVA to mares which, once infected, shed virus in most body fluids during the viraemic phase (McCollum et al., 1961) and may infect in-contacts by the respiratory route.

EAV is a single-stranded, positive-sense RNA virus recently classified in the genus Arterivirus (Cavanagh et al., 1994) which includes lactate dehydrogenase-elevating virus (Kuo et al., 1992; Godeny et al., 1993), simian haemorrhagic fever virus (Plagemann and Moennig, 1992) and the virus causing porcine respiratory and reproductive syndrome (PRRS, Conzelmann et al., 1993; Lelystad virus, Meulenberg et al., 1993a, b). The EAV virion has a diameter of 50–70 nm (Hyllseth, 1973) and consists of an isometric nucleocapsid (35 nm) surrounded by an envelope which carries ring-like surface structures with a diameter of 12–15 nm (Murphy, 1980). The nucleocapsid is composed of an infectious, polyadenylated, single-stranded genomic RNA with a size of 12.7 kb (den Boon et al., 1991) and phosphorylated core protein (N) M_r 12–14K (Hyllseth, 1973; Zeegers et al., 1976). The envelope contains a 16K non-glycosylated protein (M) and N-glycosylated proteins of M_r 25K (G_S) and 30–42K (G_L) (de Vries et al., 1992).

The major viral structural proteins G_L, M and N are encoded by open reading frames (ORFs) 5, 6 and 7, respectively; the minor envelope glycoprotein G_S is specified by ORF 2 (de Vries et al., 1992). ORFs 3 and 4 are both predicted to encode N-glycosylated membrane proteins (den Boon et al., 1991); however, the products of these ORFs have not been characterised. Virus neutralising monoclonal antibodies are directed at G_L (Balasuriya et al., 1993, 1995; Deregt et al., 1994) and recombinant G_L is currently under investigation as a subunit vaccine (Chirnside et al., 1995a) and as a diagnostic test antigen (Chirnside et al., 1995b). Should G_L prove useful as a subunit vaccine antigen, a serodiagnostic test based on an alternative virus antigen could allow differential discrimination of subunit vaccinated from other seropositive horses. This would aid the identification of naturally infected seropositive animals, in particular stallions likely to be shedding EAV in their semen, and could consequently reduce the risk of virus transmission by the venereal route.

Alternative protein candidates for use as diagnostic antigens include the EAV structural proteins G_S, M and N. EAV G_S comprises $\leqslant 2\%$ of the virion protein, has a complex glycosylation structure, and is unlikely to be a major viral antigen (de Vries et al., 1992, 1995; de Vries, 1994). Analysis of the M protein indicates that it is very hydrophobic and most likely submerged within the virus particle (de Vries et al., 1992). N comprises 35–40% of the total virion protein and is not notably hydrophobic. Fusion proteins based on EAV G_S and N react in ELISA with virus neutralising equine sera (Chirnside et al., 1995a) and rabbit, hamster and equine antisera recognise EAV N, M, G_L and G_S in immunoprecipitations and immunoblots (van Berlo et al., 1986; Iwashita and Harasawa, 1987; de Vries et

al., 1992). In this paper we present the results of cloning fragments of the EAV N protein into the bacterial expression vector pGEX to produce recombinant glutathione-*S*-transferase fusion proteins, and investigate their reactivity to equine sera in Western blots and ELISA.

2. Materials and methods

2.1. Materials

Restriction enzymes, deoxynucleoside triphosphate solutions, glutathione Sepharose 4B and pGEX-2T and -3X expression vectors were obtained from Pharmacia. Calf intestinal alkaline phosphatase, lysozyme, the Klenow large fragment of DNA polymerase, DNase I, T4 DNA polymerase and T4 DNA ligase were supplied by Boehringer Mannheim. Sequenase (Version 2.0) was obtained from U.S. Biochemical and biotin-labelled affinity-purified goat antiserum to horse IgG and streptavidin peroxidase conjugate were from Kirkegaard and Perry Laboratories Inc.

2.2. Plasmid constructs

Plasmid DNA manipulations were performed by standard methods (Sambrook et al., 1989). The prokaryotic expression vectors pGEX-2T and -3X direct the expression of cloned DNA as glutathione-S-transferase (GST) fusion proteins

Table 1
Characteristics of nucleocapsid gene constructs and fusion proteins

Fusion protein (rN)	Amino acid residue	Fusion protein size (kDa)	Derived from EAV cDNA clone	Restriction digest	Cloned into pGEX vector × RE digest
1–110	−3–110 [a]	42	c106 [b]	HindIII^{K} (12305)–HindIII^{VK} (> 12700)	3X × SmaI
1–28	−3–28 [a]	31	c106 [b]	HindIII^{K} (12305)–FspI (12399)	3X × SmaI
1–69	−3–69 [a]	36	FP70 [c]	BamH1 (12305)–RsaI (125323)	3X × BamHI EcoRI^{K}
70–89	70–89	30	FP70 [c]	RsaI (12524)–RsaI (12583)	2T × SmaI
90–110	90–110	30	FP70 [c]	RsaI (12584)–EcoRI^{VK} (> 12700)	2T × SmaI

^{K} 3′ recessed end filled in with the Klenow fragment of DNA polymerase.
^{V} Vector-derived.
[a] The negative number corresponds to additional amino acids cloned into pGEX which are not encoded by ORF 7.
[b] See de Vries et al., 1990.
[c] See Chirnside et al. 1995a.

under the control of an isopropyl-ß-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter (Smith and Johnson, 1988). EAV cDNA sequences encoding ORF 7 originating from cDNA clone 106 (de Vries et al., 1990) were ligated in frame into pGEX-3X or -2T (Table 1), transformed into *Escherichia coli* strain TG1 (Gibson, 1984), and recombinants selected on Luria broth (LB) agar containing 100 μg ampicillin per ml.

2.3. Screening for fusion proteins

Overnight cultures were diluted twenty-fold in fresh medium, grown for 2 h at 37°C and protein expression induced by addition of IPTG to 0.2 mM. After 5 h growth, 1 ml of culture was centrifuged and the bacterial pellet dissolved in 100 μl sample buffer, incubated for 5 min at 96°C and 10 μl analysed in a sodium dodecyl sulphate (SDS)–12.5% polyacrylamide gel (PAA) which was stained with Coomassie brilliant blue to visualise separated proteins. Double-stranded DNA from clones expressing an altered GST fusion protein was subjected to restriction endonuclease analysis, and recombinant plasmids sequenced with Sequenase to confirm the GST/EAV DNA junction in the correct reading frame. Sequencing was performed using the primer 5′-GGCGACCATCCTCCAAA-3′ located approximately 40 bp upstream of the pGEX multiple cloning site in addition to internal EAV-specific primers.

2.4. Purification of fusion proteins

Fusion proteins were affinity-purified using glutathione-Sepharose 4B as reported previously (Chirnside et al., 1995a).

2.5. ELISA

Optimal concentrations of reactants were determined by chequerboard titration. Immulon 3 microtitre plates (Dynatech) were coated with 0.5 μg per well of the GST or GST/EAV fusion protein and ELISAs were performed as described previously (Chirnside et al., 1995b). Equine sera at a 10^{-2} dilution had a variable background absorbance (0–0.5) caused by non-specific binding of sera to GST. Consequently the reactivity of each serum sample to GST was subtracted from that of the GST/EAV fusion protein to derive the EAV-specific absorbance value. Test sera were assayed in duplicate against each antigen and the mean absorbance at 490 nm calculated.

2.6. Immunoblotting

Following electrophoretic transfer of proteins from SDS–12.5% polyacrylamide gels onto nitrocellulose membrane immunoblots were performed as described previously (Chirnside et al., 1995b).

2.7. Virus neutralisation (VN) test

The neutralisation titres of equine sera were determined as described by Senne et al. (1985) with minor modifications (Chirnside et al., 1995b).

2.8. Immunisation

New Zealand white rabbits were immunised subcutaneously with 100 μg of affinity-purified GST or GST/EAV fusion protein in Freund's complete adjuvant, followed 5 weeks later by a second immunisation in Freund's incomplete adjuvant. Serum was collected at regular intervals and tested by virus neutralisation for antibodies to EAV.

2.9. Antisera

The antisera used in this study were submitted to the Animal Health Trust diagnostic laboratory during 1993. Isolate and vaccine-specific equine sera were kindly provided by Dr. Y. Fukunaga, Japan Racing Association.

3. Results

3.1. Cloning, expression and reactivity of EAV N fusion proteins

The characteristics of nucleocapsid gene fusion proteins and their relationship to each other are given in Table 1. The complete N protein fused to the carboxy terminus of GST was expressed as rN1–110; the remaining N fusion proteins contained smaller regions of the N gene. Although the recombinant proteins were expressed in quantity following IPTG induction (Fig. 1A) and could be purified by affinity gel chromatograpy on glutathione-Sepharose 4B, the yield of rN1–110 and rN1–69 following purification was reduced compared to rN1–28, rN70–89 and rN90–110. In immunoblots to recombinant N proteins both EAV seropositive and seronegative equine sera recognised GST (Fig. 1B). However only rN1–110 and rN1–69 were strongly bound by post-infection equine sera (Fig. 1B).

In order to examine the reactivities of equine sera in ELISA a panel consisting of 8 seronegative equine sera, used as controls in an EAV G_L ELISA (Chirnside et al., 1995b) were tested against each of the five rN proteins. The mean absorbance of these sera was determined, and a seronegative ELISA cut-off point calculated for each antigen (mean + 2 S.D. for each recombinant protein). In addition, 8 post-infection sera with VN titres ranging from 1.0 to 2.6 were screened to determine their reactivity to each rN antigen. In these ELISAs rN70–89 and rN90–110 failed to react specifically with the post-infection sera (Fig. 2); rN1–110 and rN1–28 reacted with 2 and 4 sera, respectively, whilst rN1–69 reacted with 6/8 of the post-infection equine sera. By comparison, all 8 post-infection sera recognised rG_L55–98 in ELISA.

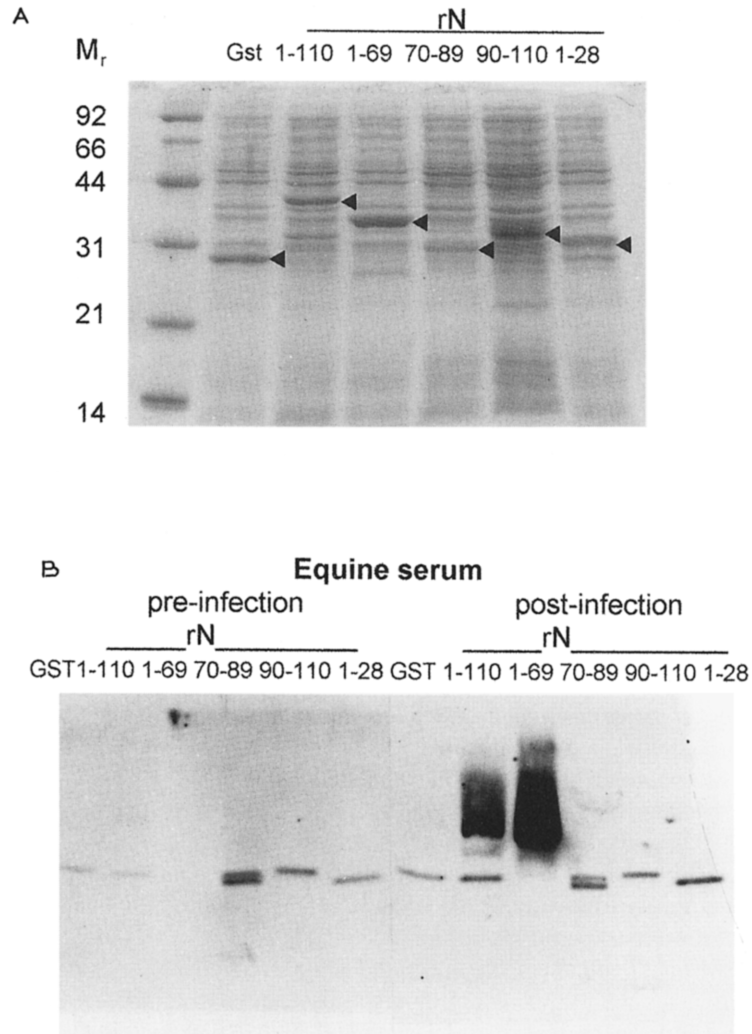

Fig. 1. (A) Expression of N fusion proteins. Bacterial lysates were run through SDS–12.5% polyacrylamide gels and stained with Coomassie brilliant blue R250. The GST lane contains lysate from pGEX-3X transformed bacteria. GST or rN fusion proteins are indicated by an arrow in each lane. Further details of the rN proteins and their cloning are shown in Table 1. The sizes of marker proteins (M_r) analysed in the same gel are indicated. (B) Immunoblots of purified fusion proteins to a pre- and post-EAV infection serum sample.

Antisera from rabbits immunised with rN1–110, rN1–69 and rN1–28 all recognised GST in ELISA and immunoblots so strongly that EAV-specific reactivity was masked (not shown). Anti-rN sera failed to neutralise EAV in VN tests in contrast to both rabbit and equine anti-rG_L55–98 sera which exhibit VN antibody activity (Chirnside et al., 1995a).

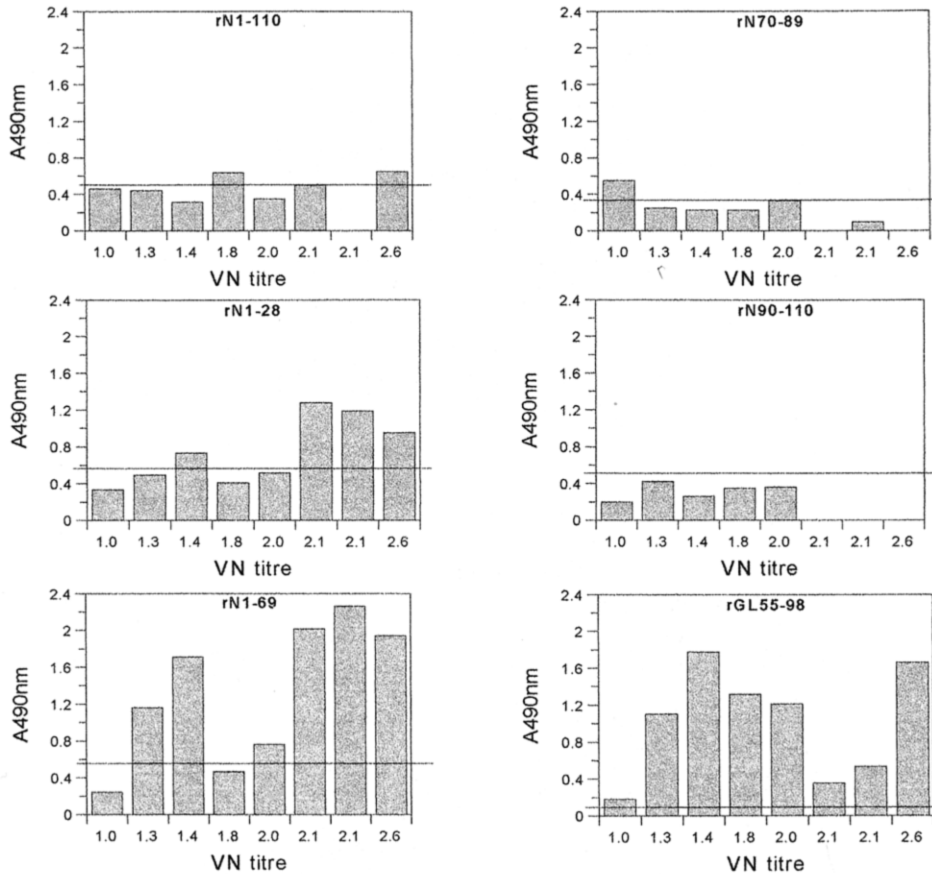

Fig. 2. ELISA absorbance values of equine sera to recombinant N proteins. ELISA plates were coated with 0.5 μg per well of purified fusion protein (rN) or GST. The GST absorbance was subtracted from the rN absorbance to derive an EAV-specific value and each bar represents the mean value from two replicates of each serum. The antigen used in ELISA is shown above each graph; the x and y axes show the $\log_{10}$ VN titre and ELISA absorbance value, respectively, for the test sera. Cut-off points determining ELISA seropositivity for each antigen, calculated from the absorbance values of 8 VN – control sera (see text), are shown as a horizontal line on each graph: rN1–110 = 0.592; rN1–28 = 0.582; rN1–69 = 0.483; rN70–89 = 0.294; rN90–110 = 0.407; rG_L55–98 = 0.100.

3.2. Recombinant N proteins as serodiagnostic antigens

The results of ELISAs with rN1–69 and rN1–28 were compared to results obtained in the EAV VN test and rG_L 55–98 ELISA (Chirnside et al., 1995b) using a panel of 47 equine sera. The panel comprised 8 VN – field sera and 8 post-infection samples as internal test controls, sera from 7 horses pre- and two weeks post-vaccination (Artervac, Fort Dodge Laboratories), 2 VN + post-vaccination samples, sera raised to three specific EAV isolates (Bucyrus, 84-KY-A1,

Table 2
Comparison of VN and ELISA tests

Sera	VN test Log_{10} titre [a]		ELISA test result ($A_{490 nm}$)					
			rG$_L$55–98 [b]		rN1–69 [c]		rN1–28 [d]	
Negative controls (n = 8)								
7	0		–		–		–	
1	0		–		–		+	
Positive controls (n = 8)								
8	**1.8–2.7**		+		+		+	
Vaccination samples	pre	post	pre	post	pre	post	pre	post
33745	0	0.3	0	**1.544**	0.132	**0.664**	0.230	**0.340**
33746	0	0.45	**0.111**	**1.886**	**0.353**	**0.967**	**0.344**	**0.580**
33747	0	0.52	**0.172**	**3.346**	0.168	**2.427**	0.256	**1.212**
33962	0	0	**0.108**	**2.252**	0.157	**0.884**	0.170	**0.387**
33963	0	**0.9**	0.015	**3.582**	0.117	**2.144**	0.145	**0.997**
33964	0	0	0.076	**2.446**	0.156	**1.572**	0.175	**0.851**
33435	0	0.3	0	**1.774**	**0.348**	**1.452**	0.286	**0.491**
35097	nd	**1.5**	nd	**3.342**	nd	**3.226**	nd	**2.026**
35098	nd	**1.5**	nd	**3.496**	nd	**3.441**	nd	**1.908**
Isolate specific sera								
Bucyrus	**3.1**		**3.740**		**3.249**		**1.130**	
84-KY-A1	**2.5**		**1.869**		**0.888**		0.288	
Wroclaw-2	**2.2**		**1.550**		**0.424**		0.276	
Arvac	**2.5**		**1.374**		**0.422**		**0.319**	
Killed Bucyrus	**1.9**		**3.193**		**1.117**		**0.620**	
Conflicting test results								
31539	0		**2.208**		**0.899**		0.289	
31571	0		**0.190**		0.147		0.174	
31577	0		0		**0.314**		**0.662**	
31578	0		**0.170**		**0.215**		**0.315**	
31658	0		**1.199**		0.148		0.210	
31724	0		**1.898**		**1.308**		**0.406**	
31736	0		**0.222**		0.101		0.148	
31746	0		**0.668**		**0.250**		**0.356**	
31568	**0.9**		0.071		0.163		0.140	
31581	**0.975**		**0.106**		**0.215**		0.176	

Boldface represents seropositive results in VN or ELISA test; pre = pre-vaccination serum sample; post = post-vaccination serum sample; nd = not determined.

[a] Log_{10} VN antibody titre ≥ 0.6 is deemed seropositive in the EAV VN neutralising test.

[b] The cut-off value to determine seropositive status was taken as (mean + 2 S.D.) of the 8 VN negative control sera (positive ≥ 0.085).

[c] The cut-off value to determine seropositive status was taken as (mean + 2 S.D.) of the 8 VN negative control sera (positive ≥ 0.177).

[d] The cut-off value to determine seropositive status was taken as (mean + 2 S.D.) of the 8 VN negative control sera (positive ≥ 0.308).

Wroclaw-2), to the live virus vaccine (Arvac; an attenuated Bucyrus preparation) and also to a formalin inactivated virus vaccine preparation (Killed Bucyrus). In addition the panel included several field sera which had previously given conflict-

ing results in EAV VN and rG_L55–98 ELISA tests in order to confirm their serum antibody status. The results of these ELISAs and VN tests are shown in Table 2.

Seropositive cut-off values to each antigen were calculated using 8 VN-field sera (Table 2). All were ELISA – to both rG_L55–98 and rN1–69, with only 7 sera ELISA – when tested against rN1–28; serum 32278 had an $A_{490\ nm}$ 0.310 which was marginally above the cut-off value (0.308) for this antigen. All eight post-infection, VN + sera were ELISA + to rN1–28, 1–69 and rG_L55–98.

The VN and rG_L55–98 ELISA tests gave conflicting results with sera from horses following the administration of one dose of a killed vaccine preparation (Artervac, Fort Dodge Laboratories). Out of 7 paired sera, only one (33963; Table 2) seroconverted in the VN test (titre $\geqslant 0.6$) following vaccination with 3 additional sera having demonstrable VN antibody (0.3–0.52) following vaccination, although not high enough to score as VN + . By comparison, all 7 sets of paired serum samples seroconverted in ELISA to rG_L55–98, rN1–69 and rN1–28 following vaccination. The strongest absorbance changes were seen with rN1–69 and rG_L55–98; the mean absorbance rises following vaccination were 2.335 ± 0.765, 1.240 ± 0.690, and 0.465 ± 0.352 for rG_L55–98, rN1–69 and rN1–28, respectively. Contrary to the results of VN testing, serum 33746 was ELISA + to all three antigens prior to vaccination. The 3 EAV isolate-specific sera and 2 EAV vaccine antisera (Arvac, Killed Bucyrus) neutralised the Bucyrus strain of EAV in VN tests and were ELISA + to both rG_L55–98 and rN1–69. However, only the 3 "Bucyrus derived" antisera recognised rN1–28.

The sera which exhibited conflicting rG_L55–98 ELISA and VN test results comprised 8 VN – and 2 VN + field sera, none of which were from vaccinated horses. In the VN – group, 4 sera were ELISA + to 2 recombinant antigens derived from different viral proteins. The remaining 4 sera were ELISA + to only one viral protein antigen, either N or G_L. Of the 2 VN + sera in this group, 31568 was ELISA – to all 3 recombinant antigens, and 31581 ELISA + to rG_L55–98.

4. Discussion

We have previously demonstrated that following infection, virus neutralising equine sera recognise G_L fusion proteins strongly and G_S and N fusion proteins inconsistently and poorly (Chirnside et al., 1995a). In the present study the reactivity of a panel of recombinant fusion proteins, comprising complete or small parts of EAV ORF 7, has been used to define the region within amino acids 1–69 as the most immunoreactive region of the viral nucleocapsid protein.

Post infection, seropositive equine sera recognised rN1–110 and rN1–69 in immunoblots and rN1–110, rN1–69 and rN1–28 in ELISA. These constructs contain the amino terminus of N, the loss of which in rN70–89 and rN90–110 produces unreactive proteins in both immunoblots and ELISAs. The poor reactivity of rN1–110 in ELISA could be due to improper folding of the N protein within this construct, as VN sera recognised it fairly strongly in immunoblots. The

complete failure of rN1–28 to react with VN sera in immunoblots, and very poorly in ELISA, may be due, at least in part, to a linear epitope spanning through and beyond amino acid residue 28. In both ELISA and immunoblots the most reactive protein was rN1–69 and although more exhaustive testing is required, these results indicate that the immunoreactive epitope of N is contained within amino acids 1–69 and that the region spanning residue 28 is important for reactivity with equine sera.

The N gene of EAV is highly conserved between isolates (Chirnside et al., 1994) so the failure of specific antisera to the Wroclaw-2 and 84-KY-A1 isolates to react with rN1–28 may be due to differences in the immune response of individual animals rather than to antigenic variation.

The reactivity of both rN1–69 and rG_L55–98 in ELISA both correlate well with the presence of VN antibody in horse sera. Recent evidence (Balasuriya et al., 1993, 1995; Deregt et al., 1994; Chirnside et al., 1995a, b) indicates that the major neutralising epitope of EAV is located on G_L. Due to its location, protein characteristics and lack of potential glycosylation sites it was unlikely that N would be the target for neutralising antibodies and the failure of N fusion proteins to induce a VN response in rabbits supports this prediction.

On the basis of our comparison to the EAV VN test results rN1–69 and rG_L55–98 are the antigens of choice for use as serodiagnostic antigens. These ELISA antigens confirmed the presence of EAV-specific antibodies in all the control VN +, post-infection VN + and EAV-specific equine sera tested and were capable of identifying vaccine induced seroconversion in the absence of a detectable VN antibody response. Additionally, rN1–69 ELISAs on sera with conflicting VN and rG_L ELISA confirmed 4/7 rG_L ELISA + results correctly. One feature of both the rG_L and rN ELISAs is their ability to detect ELISA + VN – samples, both post-vaccination and from amongst field samples submitted for diagnosis. This appears due to an increased sensitivity of the ELISAs over the VN test, which detects the sub-population of antibodies neutralising EAV from the total pool of equine antibodies reactive to viral proteins. At the current time the rG_L55–98 and rN1–69 ELISAs appear suitable for pre-screening samples prior to performing a confirmatory VN test on ELISA + samples. This could immediately accelerate both the clearing of horses with an EAV –, and reporting of horses with an EAV +, antibody status. Only more widespread use of these antigens in ELISA will confirm their suitability as stand-alone diagnostic reagents, or as replacements for the VN test.

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